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35. The method of claim 15 wherein said interferon is selected from the group consisting of IFNα IFNβ and IFNγ.

36. The method of claim 15 wherein said growth factor is G-CSF or GM-CSF.

37. The method of claim 15 wherein said interleukin is selected from the group consisting of IL1, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL11, IL12, and IL13.

REMARKS

This case contains claims 1-26 and 34-37 with the entry of this Amendment. Amendments in the as-filed claims have been made to better claim the subject matter which Applicants regard as the invention, as suggested by the Examiner. New claim 34 is a part of as-filed claim 13 which is rewritten as dependent claim. New claims 35-37 represent dependent claims of claim 15 which have been rewritten from as-filed claim 15. Therefore, none of the amendments made herein constitutes the addition of new matter.

The Rejection under 35 USC 112, second paragraph

Claim 1 has been alleged to be vague and indefinite based on the usage of the term, "nitric oxide modifying agent". Applicants point out that the definition of this term is clearly provided in the Specification, page 3, line 13 to page 4, line 16, and page 6, lines 3-16. As described herein, a nitric oxide modifying agent is a molecule which is capable of increasing endogenous NO levels, or NO production, or reducing NO quenching, or modifying the redox state of NO to a more physiologically efficacious state, for example. Accordingly, the NO modifying agents include NO gas itself, NO donors, and agents such as certain cytokines. Furthermore, the Specification states clearly what the intended effects of the NO modifying agents are, in contrast to the Examiner's allegation. The NO modifying agents are defined as agents which are capable of facilitating an NO effect on the parasite and the definition of the NO effect is further provided at page 3, lines 17-30.

Claim 15 has been alleged to be indefinite because of the symbols used in parenthesis such as IFN, G-CSF, IL, and GM-CSF. Applicants submit that these symbols have been universally

accepted as standard symbols and used as such in the art for a long time. See for example Molecular Biology of the Cell, Alberts et al. (eds) 1994, Garland Publishing, Inc. New York & London. A person of ordinary skill in the art can readily understand the meaning of each symbol without any ambiguity.

Claim 16 has been alleged to be confusing. Claim 16 has been amended for clarity. Amended claim 16 defines the agent as being a combination of a nitrothiol and another compound which is selected from a cytokine or a chemotherapeutic agent.

The basis of the claim rejection under 35 USC 112, second paragraph, have been addressed by either amending or deleting the claim language as shown above in the Claims Section. Accordingly, claims as amended are considered to be definite to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Therefore, withdrawal of the rejection is requested.

The Rejection under 35 USC 112, first paragraph

Claims 1-26 stand rejected under 35 USC 112, first paragraph as allegedly not providing enablement for a method of prophylaxis of uninfected animals by parasitic intracellular or extracellular protozoans, including the Plasmodium species. Applicants respectfully traverse this rejection.

Applicants argue that the as-filed Specification does provide support for a method of prophylaxis of uninfected animals as well as for a method of treatment of infection in animals against protozoan parasites. Example 8, at page 27, describes a study which shows the inverse relationship between malaria severity and nitric oxide production. Certain markers of NO production were measured in children with or without malaria. Healthy controls and asymptomatic individuals showed high urinary and plasma nitrate and nitrite levels and leukocyte NOS2 compared to the subjects with malaria. This indicated that increased NO synthesis can protect against malarial infection.

Further support for the claimed method of prophylaxis of malarial infection by administering a NO modifying agent comes from a publication known in the art prior to the filing date of the present application. Anstey et al. [*Transactions of the Royal Society of Tropical Medicine and Hygiene* (1997), 91:238-240, attached hereto as Exhibit A] reported that healthy children who were shown to have high levels of NO did not have malaria or the symptoms of malaria notwithstanding being resident in a high risk area. This clearly indicates that high NO levels are prophylactic in that where NO levels are elevated in at risk individuals, the risk of contracting malaria is substantially reduced.

Based on the foregoing, Applicants submit that the instant Specification provides enablement for a method of prophylaxis as well as treatment of malarial infection in animals by administering a NO modifying agent. A person of ordinary skill in the art can make and use the claimed invention based on the description provided in the Specification combined with the knowledge available in the art without undue experimentation. Thus, withdrawal of the rejection under 35 USC 112, first paragraph is respectfully requested.

The Rejection under 35 USC 102

Claims 1-6, 9-13, 15, 17-19, 22-23, and 26 are rejected under 35 USC 102(b) as allegedly anticipated by Seguin et al. (*The Journal of Experimental Medicine*, 1994). Applicants respectfully traverse this rejection.

Seguin et al. describes results showing that both CD8+ T cells and IFNγ are important components in the regulation of iNOS in liver which might contribute to the protective response of mice immunized with irradiated malaria sporozoite.

In contrast, the claimed method of preventing or treating malarial infection with a NO modifying agent is based on the inventors' finding that NO production retards parasitic growth. Therefore, the methods of treating or preventing parasitic infection can be applied at any stage of its life cycle. Because the key factor of the claimed methods lies in modulating NO production, any agent capable of facilitating NO production is useful in practicing the invention as disclosed in the Specification.

The claimed invention cannot be anticipated by Seguin et al. Results obtained in immunized mice cannot anticipate results obtained in non-immunized humans. The study by Seguin et al. involved multi-components which may or may not have any cause-effect relationship. The authors first immunized mice with irradiated parasites and tested them for protection against subsequent sporozoite challenge. This challenge was then combined with substrate inhibitors of NOS or Mab of IFNγ. Based on the results of these manipulations, it was speculated that the development of exoerythrocytic stages of malaria was prevented because of the induction of NO synthase. Seguin et al. did not establish the direct link that NO production inhibits parasite growth, as was disclosed in the present application. Applicants point out that Seguin et al. at best provided mere speculation as to the role of NO in malarial infection, which was known at the time. There is no teaching in the cited reference of the administration of a NO modifying agent for treating or preventing malarial infection. Seguin et al. related only to liver stage malaria not to blood stage malaria. Furthermore, this reference only considered the anti-parasitic effect of NO and not the anti-disease effects of parasitic infection. What the instant invention shows is that increased levels of NO prevents disease development in non-immunized humans. Anticipating references must teach each and every element of the claimed invention. Therefore, the claimed invention cannot be anticipated by Seguin et al.

The Rejection under 35 USC 103

Claims 1-17, 9-13, 15 and 17-26 are rejected under 35 USC 103 (a) as allegedly unpatentable over Kremsner et al. in view of Liew et al. Claims 1, 8, 14, and 16 are further rejected under 35 USC 103(a) as allegedly unpatentable over Seguin et al. or Liew et al. in view of Stamler et al. Applicants respectfully traverse these rejections.

Kremsner et al. describes the production of NO induced by a cytokine and that NO is toxic in vitro to plasmodium falciparum. There is no teaching or suggestion of the administration of a NO modifying agent to treat or prevent malarial infection especially at the blood stage. In fact, Kremsner et al. reported that NO production contributed to the pathogenecity of the disease. This clearly teaches away from the claimed invention. Thus, a person of ordinary skill cannot derive any motivation from the Kremsner reference to make the claimed method of treating malarial infection with a NO modifying agent.

Liew et al. teaches that murine macrophages produce high levels of nitric oxide upon activation by IFNγ, IFNα and LPS which were efficient in killing *Leishmania major, in vitro*. There is no mention of parasites which cause malaria. There is no suggestion that an NO modifying agent can be used to treat or prevent malarial infection.

Stamler et al. is a reference which provides biochemical characteristics of nitric oxide and NOS. It simply describes the reactivity between nitric oxide and certain plasma protein thiols which modulates plasma levels of S-nitrosothiols. There is no teaching or suggestion of the role of NO or NOS in malarial infection. There is no suggestion that a NO modifying agent can be used to treat or prevent malarial infection.

A rejection for obviousness over a combination of references cannot be sustained unless motivation to combine the teachings therein can be found within the references themselves. None of the cited references provide such motivation. Seguin et al. merely supported the suspicion, that there may be some link between the NO levels and malarial infection. Liew et al. described the role of NO in a unrelated disease, *Leishmania major, in vitro*. Stamler et al. suggests no disease conditions which can be modulated by administering the NO modifying agents. Kremsner et al. reported controversial results which teach a person of ordinary skill away from attempting to make the claimed methods.

In summary, a person of ordinary skill cannot derive motivation from the cited references to combine them to make and/or use the claimed invention.

In view of the foregoing amendments and remarks, it is submitted that the present application is in condition for allowance, and passage to issuance is respectfully requested.

If there are any additional issues related to patentability, the courtesy of a telephone interview is requested, and the Examiner is invited to call to arrange a mutually convenient time.

This amendment is accompanied by a Petition for Extension of Time (three months) and a check in the amount of $445.00 as required under 37 C.F.R. 1.17. It is believed that this

amendment does not necessitate the payment of any additional fees under 37 C.F.R. 1.16-1.17. If the amount submitted is incorrect, however, please deduct from Deposit Account No. 07-1969 the appropriate fee for this submission and any extension of time required.

Respectfully submitted,

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COPY

Correspondence

Nitrate levels in malaria

We read with interest the recent studies by Kremsner *et al.* (1996: *Transactions*, 90, 44) and Al Yaman *et al.* (1996: *Transactions*, 90, 270) extrapolating nitric oxide (NO) production from plasma levels of the NO metabolites nitrate plus nitrite (NO_x) in children with malaria. We believe, however, that plasma NO_x levels in these studies cannot be reliably interpreted without correction for confounding variables that influence the levels of NO metabolites, and without data from control groups.

Renal impairment and enhanced renal tubular NO_x absorption significantly increase plasma NO_x levels in disease states, including malaria, without necessarily implying increased NO production (Mackenzie *et al.*, 1996: *Clinical Chemistry*, 42, 440; Anstey *et al.*, 1996: *Journal of Experimental Medicine*, 184, 557). Sixty to 73% of a nitrate load is excreted renally (Green *et al.*, 1981: *Proceedings of the National Academy of Sciences of the USA*, 78, 7764; Westfelt *et al.*, 1995: *British Journal of Pharmacology*, 114, 1621). Plasma creatinine and serum NO_x were closely correlated in a recent study of unselected intensive care unit patients (Mackenzie *et al.*, *loc. cit.*). Nitrate is retained and plasma NO_x markedly elevated in otherwise healthy humans with renal impairment (Strand *et al.*, 1995: *Endothelium*, 3, supplement, s98). Although not as severe as in adults, renal impairment occurs in a significant proportion of children with severe and complicated malaria. In our recent study of Tanzanian children with malaria, renal impairment (conservatively defined as plasma creatinine >2 SD above mean creatinine level for age) was found in 28% of children with cerebral malaria (21% of those with complete recovery and 39% of those with a course complicated by death or neurological sequelae) (Anstey *et al.*, *loc. cit.*). In addition, we found that the fractional excretion of NO_x (the proportion of filtered NO_x that is excreted) was decreased in clinical malaria, being lowest in those with fatal cerebral malaria. In cerebral malaria, therefore, not only was glomerular filtration of nitrate reduced (as measured by increased plasma creatinine), but the filtered nitrate was more avidly reabsorbed in the renal tubules. Both of these factors acted to increase uncorrected plasma NO_x levels in the cerebral malaria group compared with the group with uncomplicated malaria. Like Al Yaman *et al.* (*loc. cit.*), we found that uncorrected plasma NO_x levels were higher in children with fatal cerebral malaria relative to the cerebral malaria group with complete recovery. However, this apparent elevation disappeared once NO_x levels were corrected for renal impairment (expressed as plasma NO_x:creatinine ratio), with mean plasma NO_x:creatinine in the fatal cerebral malaria group being the lowest of all groups with malaria. This suggests that impaired nitrate excretion rather than increased NO synthesis was the cause of the relative elevation of uncorrected plasma NO_x in fatal cerebral malaria. In the Papua New Guinea study, the association between depth of coma and uncorrected plasma NO_x levels probably reflected a correlation between the duration and severity of coma and the severity of renal impairment (and thus NO_x retention). The validity of the correction for renal impairment as a measure of NO production is supported by the very close correlation between mean plasma NO_x:creatinine ratios, mean urinary NO_x excretion, and mean leucocyte immunoblot band density of type 2 inducible nitric oxide synthase (NOS2) antigen in Tanzanian children (Anstey *et al.*, *loc. cit.*).

Because the volume of distribution of NO_x ($VdNO_x$) approximates extracellular fluid volume, plasma NO_x levels will be increased and NO formation overestimated in disease states characterized by extracellular fluid volume concentration (Zeballos *et al.*, 1995: *Circulation*, 91, 2982). Dehydration occurs commonly in children with severe malaria (Waller *et al.*, 1995: *Clinical Infectious Diseases*, 21, 577; English *et al.*, 1996: *Archives of Diseases of Childhood*, 74, 201). It is likely therefore that $VdNO_x$ was reduced in the children with severe malaria in the studies of both Kremsner *et al.* (*loc. cit.*) and Al Yaman *et al.* (*loc. cit.*), and that NO formation was further overestimated in these children.

Although the Gabonese (Kremsner *et al.*, *loc. cit.*) and Papua New Guinean (Al Yaman *et al.*, *loc. cit.*) studies did not control for dietary nitrate ingestion, we agree with both groups that, because of the prolonged duration of fasting in most of the children with severe and cerebral malaria, dietary nitrate ingestion is unlikely to have contributed significantly to their admission NO_x levels. However, without dietary control or fasting, it is difficult to extrapolate NO production in the Gabonese study from the convalescent NO_x levels reported in both the uncomplicated and severe malaria groups.

Interpretation of the data of both Kremsner *et al.* (*loc. cit.*) and Al Yaman *et al.* (*loc. cit.*) is also difficult because of the absence from both studies of NO_x levels from fasting healthy control children from the same study sites. In Tanzania, we found that, in fasting healthy control children, plasma NO_x levels (with or without correction for renal impairment) and urine NO_x excretion were both significantly higher than in children with either uncomplicated or cerebral malaria. Surprisingly, NOS2 was expressed in blood mononuclear cells from all healthy controls tested but in only one child with cerebral malaria (Anstey *et al.*, *loc. cit.*). This constitutive expression of NOS2 by blood mononuclear cells is very unusual in healthy American adults (St. Clair *et al.*, 1996: *Journal of Experimental Medicine*, 184, 1173). The use of urine NO_x excretion, renally-corrected plasma NO_x (Mackenzie *et al.*, 1994: *Lancet*, 344, 410; Anstey *et al.*, *loc. cit.*), and immunoblotting for leucocyte NOS2 antigen expression allowed us to demonstrate increased NO production in asymptomatic parasitaemic malaria patients and suppression of systemic NO production in those with uncomplicated and cerebral malaria. There was an inverse correlation between malaria disease severity and NO production and leucocyte NOS2 expression (Anstey *et al.*, *loc. cit.*).

We agree with Kremsner *et al.* (*loc. cit.*) that NO appears to be protective against malaria, but do not agree that either group's data in their current form can be used to support the notion of increased NO production in severe and cerebral malaria. If the studies of Kremsner *et al.* (*loc. cit.*) and Al Yaman *et al.* (*loc. cit.*) had included a group of fasting healthy controls and if they had corrected each plasma NO_x level for renal impairment, we believe it is possible that they too would have found an inverse correlation between malaria disease severity and NO production in children in both Gabon and Papua New Guinea. It would thus be of great interest, if sera from both studies were still available, to measure creatinine levels, to enable their NO_x results to be expressed in renally-corrected form.

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EXHIBIT A